RECK-Mediated Suppression of Tumor Cell Invasion Is Regulated by Glycosylation in Human Tumor Cell Lines

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Abstract

RECK, a glycosylphosphatidylinositol (GPI)–anchored glycoprotein, negatively regulates matrix metalloproteinases (MMP), such as MMP-9, and inhibits tumor invasion and metastasis. The predicted amino acid sequence of human RECK includes five putative N-glycosylation sites; however, the precise biochemical role of glycosylated RECK remains unknown. In this study, we examined the link between glycosylation and the function of RECK in human tumor cell lines. RECK protein was glycosylated at Asn^{86}, Asn^{200}, Asn^{297}, and Asn^{352} residues but not at the Asn^{39} residue in HT1080 cells. Although the glycosylation of these asparagine sites did not play a role in the cell surface localization of RECK as a GPI-anchored protein, the glycosylation of RECK Asn^{297} residue was involved in the suppression of MMP-9 secretion and Asn^{352} residue was necessary to inhibit MMP-2 activation. Moreover, RECK-suppressed tumor cell invasion was reversed by inhibiting glycosylation at Asn^{86}, Asn^{297}, and Asn^{352} residues of RECK. Thus, these findings indicate that glycosylation mediates RECK suppression of tumor cell invasion by multiple mechanisms such as suppressing MMP-9 secretion and inhibiting MMP-2 activation. (Cancer Res 2005; 65(16): 7455-61)

Introduction

One well-known characteristic of malignant tumors is their ability to invade tissues and generate metastasis. Due to the capacity of degradative enzymes such as proteases and glycosidases to degrade extracellular matrix (ECM) proteins, they are important components of this process (1–5). Among these enzymes, matrix metalloproteinases (MMP) have been the focus of much anticancer research (6). MMPs mediate both the ECM, as well as basement membrane degradation during the early stages of tumorigenesis, contributing to the formation of a microenvironment that promotes tumor growth (7, 8). MMP activity is regulated at three main levels; transcription, proenzyme activation, and inhibition. Malignant tumors have developed strategies to circumvent these regulatory mechanisms and to generate the uncontrolled proteolytic activity that accompanies cancer development and metastasis.

MMP activity is blocked by general inhibitors, such as α_2-macroglobulin, which are present in the plasma and tissue fluids; MMP activity is also blocked by more specific inhibitors such as tissue inhibitors of metalloproteinases (TIMP; ref. 9). Four human TIMPs have been identified, which are anchored in the ECM or secreted extracellularly. These TIMPs bind MMPs tightly and noncovalently in 1:1 stoichiometric complexes (9). The net balance between protease and inhibitor activities determines the proteolytic potential of tumors; decreases in TIMP levels are generally correlated with tumorigenesis (10). Several recently described proteins are novel MMP inhibitors, and some contain domains that are homologous to the TIMP inhibitory domains. For example, tissue factor pathway inhibitor-2 (TFPI2) is a serine protease inhibitor that can function as an MMP inhibitor (11, 12). The procollagen COOH-terminal proteinase enhancer releases a COOH-terminal fragment that is similar to the inhibitor domain of TIMPs, and this fragment possesses significant MMP inhibitory activity (13). RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a cell surface MMP inhibitor and a key regulator of ECM integrity and angiogenesis (14, 15).

The *RECK* gene was isolated by an expression cloning strategy designed to isolate human cDNA, inducing flat reversion in *v-Ki-ras*-transformed NIH3T3 cells (14, 16). The *RECK* gene contains serine protease inhibitor-like domains and is associated with the cell membrane through a COOH-terminal glycosylphosphatidylinositol (GPI) modification (14). Although the *RECK* gene is widely expressed in various human organs, its expression is low in many tumor-derived cell lines (14, 15, 17). The amount of extracellular pro-MMP-9 and of active-MMP-2 was shown to be reduced in RECK-overexpressed tumor cells, and their invasive and metastatic potential was suppressed (14), suggesting that RECK plays a role in the regulation of MMPs and tumor malignancy.

The formation of the sugar-amino acid linkage is a crucial event in the biosynthesis of the carbohydrate units of glycoproteins (18–21). In the case of N-glycosylation, as the nascent glycoprotein enters the endoplasmic reticulum (ER), a preformed oligosaccharide known as the dolichol-phosphate precursor is attached cotranslationally to certain asparagine residues that are part of the consensus sequence Asn-Xaa-Ser/Thr (where Xaa represents any amino acid except Pro). Many of the functions of protein N-glycosylation have been described to date, including secretion to the extracellular space (e.g., heparanase; ref. 22), the promotion of protein activation (e.g., meprin A; ref. 23), and the stabilization of glycoproteins (e.g., H,K-ATPase β subunit; ref. 24). Based on the predicted amino acid sequence, human RECK contains five putative N-glycosylation sites; however, the biochemical roles played by glycosylation in the function of RECK has not yet been investigated.

In this report, we show that the glycosylation of RECK is not required for its cell surface localization as a GPI-anchored protein; however, such glycosylation is important for the RECK suppression of tumor cell invasion. Our findings suggest that glycosylated RECK may be responsible for RECK-suppressed tumor cell invasion. Thus, not only the level of expression but also the glycosylation status may have important implications for the MMP inhibitory activity of RECK.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Establishment of RECK-overexpressing stable cell lines. To obtain RECK-overexpressing cell lines, we carried out gene transfection using a previously described method (25, 26) according to the manufacturer's instructions. The human *RECK* gene was kindly provided Dr. Makoto Noda (Kyoto University), and was cloned into the pZeoSV2(+) vector (Invitrogen, Carlsbad, CA). Permanent cell lines expressing RECK protein were established by transfecting pZeoSV2(+)-*RECK* into HT1080 cells followed by 50 μg/mL Zeocin (Invitrogen) selection. The cells transfected with pZeoSV2(+) vector were designated as HT1080-Zeo cells, and the clone cells expressing high levels of RECK protein were designated as HT1080-RECK cells.

Construction of RECK mutants. We substituted certain asparagine residues in the RECK protein, including five putative N-glycosylation sites, with glutamine residues by PCR site-directed mutagenesis using the technique of overlap extension (22, 27). The sets of primers used for the mutagenesis are listed in Supplementary Table S1.

Western blotting. Cells were left untreated or were treated with various concentrations of tunicamycin A (Calbiochem, La Jolla, CA) and were then lysed in lysis buffer [10 mmol/L HEPES, 142.5 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.2% NP40, 0.1% aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.2)] at 4°C with sonication (28). The lysates were centrifuged at 15,000 rpm for 15 minutes, and the amount of protein in each lysate was measured by staining the samples with Coomassie Brilliant Blue G-250 (Bio-Rad Lab., Hercules, CA). Loading buffer [42 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, and 0.002% bromophenol blue] was then added to each lysate, which was subsequently boiled for 3 minutes and electrophoresed on SDS-polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with anti-RECK (BD Biosciences, San Jose, CA) and anti-α-tubulin (Sigma, St. Louis, MO) antibodies. Detection was done with enhanced chemiluminescence reagent (Pierce, Rockford, IL).

***In vitro* glycosidase treatment.** Exponentially growing cells were lysed in B solution (0.5% SDS and 1% 2-mercaptoethanol) with sonication and boiled for 10 minutes. After centrifugation at 15,000 rpm for 15 minutes, the supernatant (90 μL) was added to 10 μL of sodium phosphate buffer [final concentration: 50 mmol/L sodium phosphate (pH 7.5) and 1 % NP40] and subsequently incubated with 2 units peptide N-glycosidase F (PNGase-F; Roche, Mannheim, Germany) at 37°C for 3 hours (22). The reaction was terminated by the addition of loading buffer; the samples were then boiled for 3 minutes, subjected to electrophoreses, and immunoblotted with anti-RECK antibody.

Phosphatidylinositol-specific phospholipase C treatment. The GPI-anchored proteins were released into the culture medium by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). To evaluate the role of glycosylation in the cell surface localization of RECK, exponentially growing cells (3×10^5 cells) in 6-well plate were washed and cultured in serum-free medium (750 μL) in the presence or absence of PI-PLC (100 milliunits) for 12 hours. The conditioned media were collected by centrifugation at 15,000 rpm for 15 minutes, and loading buffer was added to the supernatant, which was subsequently boiled for 3 minutes and electrophoresed on SDS-polyacrylamide gel. The cell lysates (200 μL) were prepared as described above. The amounts of RECK in both the cell lysates (15 μL) and the culture media (30 μL) were measured by Western blotting using anti-RECK antibody (13).

Fluorescence microscopy. To observe the cell surface localization of RECK, we constructed FLAG-tagged RECK and FLAG-tagged RECK/4NQ expression vectors and established stable clones designated as HT1080-RECK-F and HT1080-RECK/4NQ-F, respectively. The FLAG epitope was introduced between Tyr^{783} and Tyr^{784} within human RECK. For the cell surface labeling with anti-M2 antibody (Sigma), we applied a slightly modified form of the method described by Yoshimori et al. (29). The cells were incubated with the antibody for 8 hours at 4°C before fixation. After being washed with PBS, the cells were fixed and incubated with Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Inc., Eugene, OR). The cells were then washed with PBS and incubated with Hoechst33258 (1 μg/mL) for 5 minutes (22). Then, the cells were washed with PBS and were examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Gelatin zymography. Exponentially growing cells were seeded at 1×10^5 cells per well in 24-well plates (Sumilon multiwell plates, Sumitomo Bakelite Corp., Tokyo, Japan) for 12 hours. After cell attachment, the culture medium was exchanged for serum-free medium (200 μL) for 22 hours. The conditioned media were collected by centrifugation at 15,000 rpm for 15 minutes, and loading buffer without a reducing agent was added to the supernatant, which was subsequently electrophoresed on an SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was incubated twice in 2.5% Triton X-100 for 1 hour and incubated overnight in 50 mmol/L Tris-HCl (pH 7.5), 200 mmol/L NaCl, and 10 mmol/L $CaCl_2$ at 37°C. Then, the gel was stained with Coomassie brilliant blue (30). Fibronectin was monitored as the control for the secreted proteins in HT1080 cells (31), as measured by Western blotting using anti-fibronectin antibody (H-300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

***In vitro* invasion assay.** *In vitro* invasion of HT1080 cells was assessed by a previously described method (32, 33) according to the manufacturer's instructions (Becton Dickinson Labware, Bedford, MA). A BD BioCoat Matrigel Invasion Chamber (8-μm pore size; Becton Dickinson Labware) was used for the invasion assay. The chambers were placed on 24-well plates. Then, a tumor cell suspension (1×10^5 cells/0.5 mL) in serum-free DMEM was added to the upper layer, and 0.75 mL of DMEM (10% FCS) was added to the lower layer. After incubation for 22 hours at 37°C, the tumor cells were fixed in methanol, and the cells on the upper surface of the membrane were completely removed by wiping with cotton swabs. The cells on the lower surface of the membrane were stained with crystal violet. To extract the dye, 1 mL of 1 mmol/L HCl in 30% aqueous ethanol was added into each well. The absorbance was measured at 540 nm.

Results

Expression of glycosylated RECK in cultured human cell lines. We first analyzed RECK expression in several human cell lines using Western blotting. As shown in Fig. 1*A*, the expression levels of RECK were high in normal cells, such as WI-38 cells (normal lung fibroblasts) and human umbilical vein endothelial cells (HUVEC); however, the levels of RECK were very low among solid tumor-derived cell lines, including HT1080 (fibrosarcoma), HeLa (cervical carcinoma), and HepG2 (hepatoma) cells. In leukemia cell lines, high levels of expression were observed in Jurkat (T-cell leukemia) and U937 (promonocytic leukemia) cells, whereas the expression levels in K562 (erythroleukemia) and HL-60 (promyelocytic leukemia) cells were low (Fig. 1*A*). Interestingly, in the extracts from HUVECs and WI-38 cells, two or three forms of RECK were clearly observed (Fig. 1*A*). Because the faster migrating forms of RECK are thought to be hypoglycosylated forms of RECK, we treated cell lysates from WI-38, HUVECs, HT1080, and HeLa cells with PNGase-F *in vitro*. The sensitivity to PNGase-F, an enzyme that removes all types of N-linked oligosaccharides from glycoprotein, is an indication of N-linked protein glycosylation. As shown in Fig. 1*B*, treatment with PNGase-F eliminated the slower migrating form and gave only faster migrating forms, suggesting that endogenous RECK is expressed as both hyperglycosylated and hypoglycosylated forms in these cells. To clarify the role of the glycosylation of RECK in its function, we established RECK-overexpressing cells, designated as HT1080-RECK cells, and examined whether or not RECK is glycosylated in these cells. RECK protein was detected as two bands in the extracts from the HT1080-RECK cells (Fig. 1*C*). Treatment of HT1080-RECK cells with tunicamycin A, an inhibitor of glycosylation, resulted in a

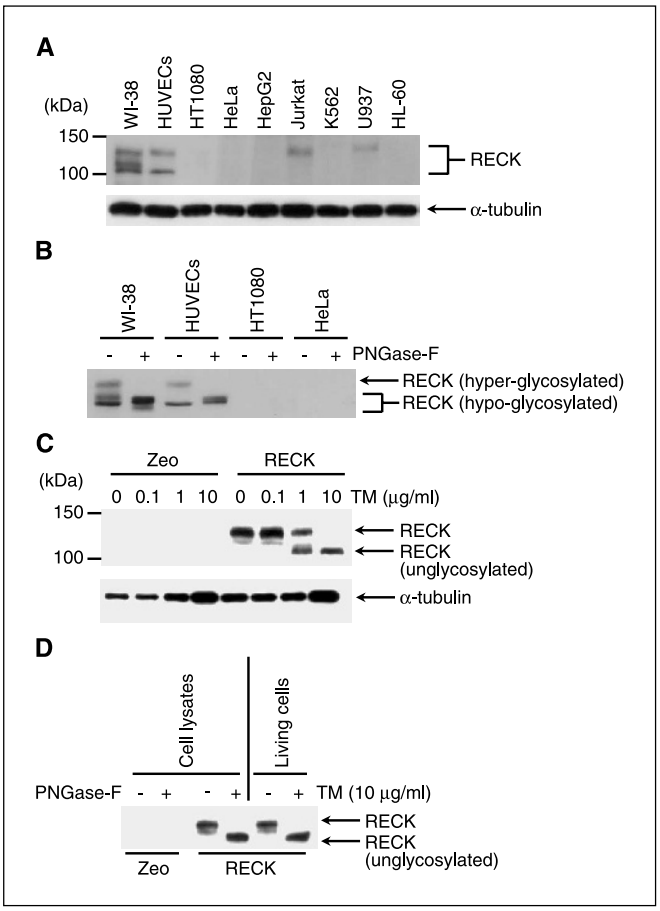

Figure 1. Glycosylation of RECK in the cells. *A,* glycosylation of endogenous RECK in cultured human cell lines. WI-38, HUVECs, HT1080, HeLa, HepG2, Jurkat, K562, U937, and HL-60 cells were lysed, and Western blotting was carried out using the indicated antibodies. *B,* deglycosylation of endogenous RECK by glycosidase *in vitro*. WI-38, HUVECs, HT1080, and HeLa cells were lysed, and aliquots of the cell lysates were incubated with or without PNGase-F for 3 hours. The samples were electrophoresed and immunoblotted with anti-RECK antibody. *C,* inhibition of RECK glycosylation by treatment with tunicamycin A (*TM*) in HT1080-RECK cells. HT1080-Zeo and HT1080-RECK cells were treated with various concentrations of tunicamycin A for 24 hours. Aliquots of the cell lysates were immunoblotted with the indicated antibodies. *D,* deglycosylation of RECK from HT1080-RECK cells by glycosidase *in vitro*. HT1080-Zeo and HT1080-RECK cells were lysed, and aliquots of the cell lysates were incubated with PNGase-F for 3 hours. The samples were electrophoresed and immunoblotted with anti-RECK antibody. The lysates from tunicamycin A–treated or untreated HT1080-RECK cells were also analyzed.

reduction in the size of RECK, as detected by electrophoresis and Western blotting using anti-RECK antibody (Fig. 1*C*). Tunicamycin A (1 μg/mL) resulted in a partial inhibition of glycosylation, whereas 10 μg/mL of tunicamycin A inhibited glycosylation completely (Fig. 1*C*). To further examine whether or not RECK was indeed glycosylated in the cells, we employed the method of glycosidase treatment with PNGase-F *in vitro* (Fig. 1*D*). PNGase-F treatment generated a band of ~100 kDa, a finding which was consistent with the molecular weight deduced from the amino acid sequence of RECK (deglycosylated form). Both of the two slower migrating bands of RECK were changed into only the faster migrating form by treatment with tunicamycin A or with PNGase-F *in vitro*, suggesting that both of these bands of RECK were hyperglycosylated forms. Thus, these findings indicate that RECK is expressed in hyperglycosylated forms in cultured human cell lines such as WI-38 and HUVECs, as well as in HT1080-RECK cells.

Glycosylation of RECK at four putative glycosylation residues. The primary amino acid sequence of human RECK contains five predicted N-linked glycosylation consensus sequences: Asn^{39}, Asn^{86}, Asn^{200}, Asn^{297}, and Asn^{352} residues (Fig. 2*A*). To identify the glycosylation site(s) within RECK, we constructed several mutant forms of the protein, in which the asparagine residues were replaced with glutamine residues. The electrophoretic migration of RECK prepared from HT1080-RECK/N86Q, HT1080-RECK/N200Q, HT1080-RECK/N297Q, and HT1080-RECK/N352Q cells was slightly faster than that of RECK prepared from HT1080-RECK cells, which was suggestive of a size reduction among mutant RECK proteins (Fig. 2*B*). On the other hand, no size reduction among RECK proteins took place in HT1080-RECK/N39Q cells, indicating that the Asn^{39} residue was not glycosylated (Fig. 2*B*). Asn^{209} is not located within a glycosylation consensus sequence and was therefore mutated as a control. We did not observe any reduction in the size of RECK in HT1080-RECK/N209Q cells (Fig. 2*B*). Electrophoresis revealed a reduction in the size of RECK proteins from HT1080-RECK/5NQ cells (in which all five putative N-glycosylation asparagine residues located

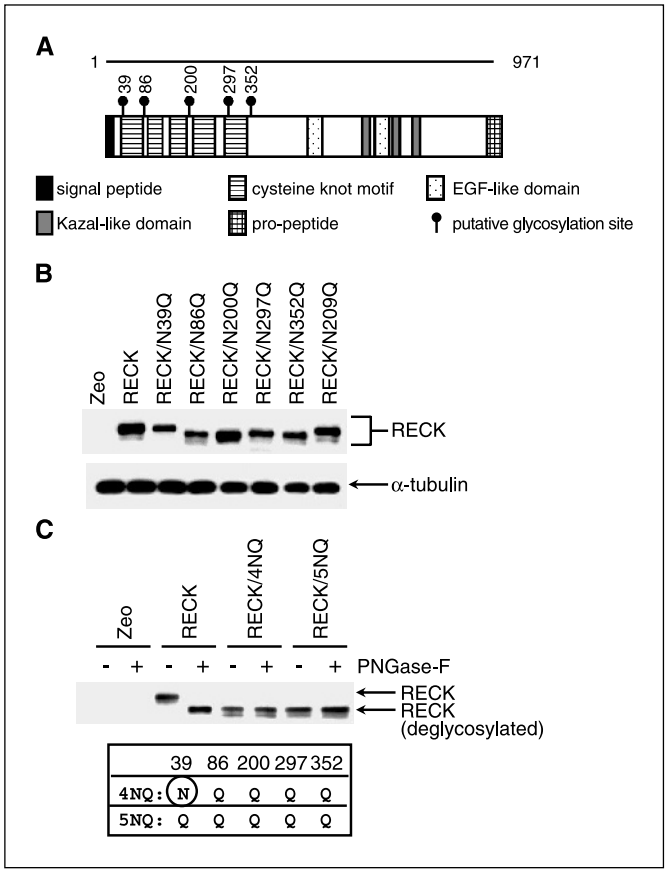

Figure 2. Glycosylation of RECK at four putative glycosylation sites in the cells. *A,* schematic diagram of human RECK. The locations of the five putative N-linked glycosylation sites (N39, N86, N200, N297, and N352) are indicated by solid circles. *B,* glycosylation of RECK at Asn^{86}, Asn^{200}, Asn^{297}, and Asn^{352} residues in the cells. HT1080-Zeo, HT1080-RECK, HT1080-RECK/N39Q, HT1080-RECK/N86Q, HT1080-RECK/N200Q, HT1080-RECK/N297Q, HT1080-RECK/N352Q, and HT1080-RECK/N209Q cells were lysed, and aliquots of the cell lysates were immunoblotted with the indicated antibodies. *C,* the Asn^{39} residue of RECK was not glycosylated in the cells. HT1080-Zeo, HT1080-RECK, HT1080-RECK/4NQ, and HT1080-RECK/5NQ cells were lysed, and aliquots of the cell lysates were treated with PNGase-F for 3 hours. The samples were immunoblotted with anti-RECK antibody.

within a consensus sequence were replaced with glutamine residues) and from HT1080-RECK/4NQ cells (in which all four N-glycosylation asparagine residues except for Asn^{39} (Asn^{86}, Asn^{200}, Asn^{297}, and Asn^{352}) were replaced with glutamine residues) (Fig. 2*C*). The molecular size of RECK/4NQ, RECK/5NQ, and PNGase-F-treated wild-type RECK was identical; moreover, the molecular sizes of RECK/4NQ and RECK/5NQ did not change following treatment with PNGase-F (Fig. 2*C*). Therefore, these results indicate that N-glycosylation does indeed occur at the Asn^{86}, Asn^{200}, Asn^{297}, and Asn^{352} residues but not at the Asn^{39} residue of RECK.

Effect of glycosylation on the cell surface localization of RECK as a glycosylphosphatidylinositol-anchored protein. Subsequently, to examine the effect of glycosylation on the cell surface localization of RECK as a GPI-anchored protein, we compared the amount of RECK in the cell lysates with that of conditioned media treated with PI-PLC, an enzyme that can cleave the GPI anchor, in wild-type RECK- and mutant RECK-expressing cells. As shown in Fig. 3*A*, treatment of HT1080-RECK cells with PI-PLC resulted in the release of RECK into the culture medium, whereas only a trace amount of RECK was observed in PI-PLC-untreated conditioned medium, indicating that RECK is a GPI-anchored protein. In RECK/N39Q-, RECK/N86Q-, RECK/N200Q-, RECK/N297Q-, RECK/N352Q-, RECK/N209Q-, RECK/4NQ-, and RECK/5NQ-expressing cells, all corresponding RECK mutants were released from the cells into the culture media following treatment with PI-PLC (Fig. 3*A*); however, only trace amounts of these mutant RECKs were observed in PI-PLC-untreated conditioned media (data not shown). Thus, these findings suggest that the glycosylation of RECK is not required for the cell surface localization of RECK as a GPI-anchored protein. To observe the cell surface localization of RECK by immunostaining using anti-FLAG antibody, we constructed expression vectors of FLAG-tagged RECK (designated as RECK-F; Fig. 3*B*) and established stable clones that overexpressed RECK-F and RECK/4NQ-F. RECK proteins from HT1080-RECK-F cells and HT1080-RECK/4NQ-F cells were expressed at identical levels, as assessed by Western blotting (data not shown). Using these cell lines, RECK-F and RECK/4NQ-F were detected at the cell surface of detergent-untreated cells (Fig. 3*C*). Thus, these results also suggest that the glycosylation of RECK is not involved in the cell surface localization of this protein.

Glycosylation of RECK Asn^{297} and Asn^{352} residues is involved in the suppression of matrix metalloproteinase-9 secretion and of matrix metalloproteinase-2 activation, respectively. We examined the effect of glycosylation on RECK activity, as based on the suppression of MMP-9 secretion and of MMP-2 activation into the culture medium evaluated by gelatin zymography and Western blotting (data not shown). As reported previously (14, 15), the overexpression of RECK was associated with a decrease in the amount of MMP-9 detected in the culture

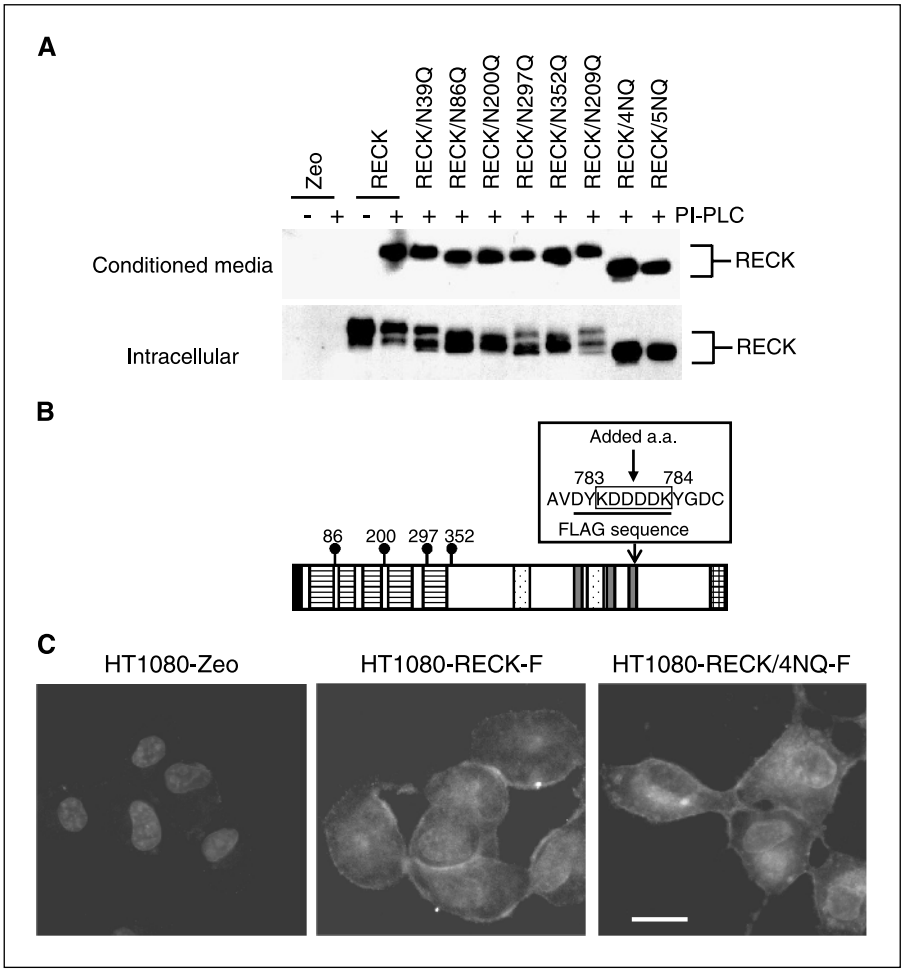

Figure 3. Effect of glycosylation on the cell surface localization of RECK. *A,* HT1080-Zeo, HT1080-RECK, HT1080-RECK/N39Q, HT1080-RECK/N86Q, HT1080-RECK/N200Q, HT1080-RECK/N297Q, HT1080-RECK/N352Q, HT1080-RECK/N209Q, HT1080-RECK/4NQ, and HT1080-RECK/5NQ cells were washed and were left untreated or were treated with 100 milliunits per well PI-PLC for 12 hours in serum-free media. Samples of the conditioned media (*top*) and aliquots of the cell lysates (*bottom*) were electrophoresed and immunoblotted with anti-RECK antibody. *B,* schematic representation of FLAG-tagged RECK (RECK-F). *C,* effect of RECK glycosylation on cell surface localization. HT1080-Zeo, HT1080-RECK-F, or HT1080-RECK/4NQ-F cells were stained with anti-FLAG antibody and Hoechst33258, and then the cells were observed by fluorescence microscopy. *Bar,* 20 μm.

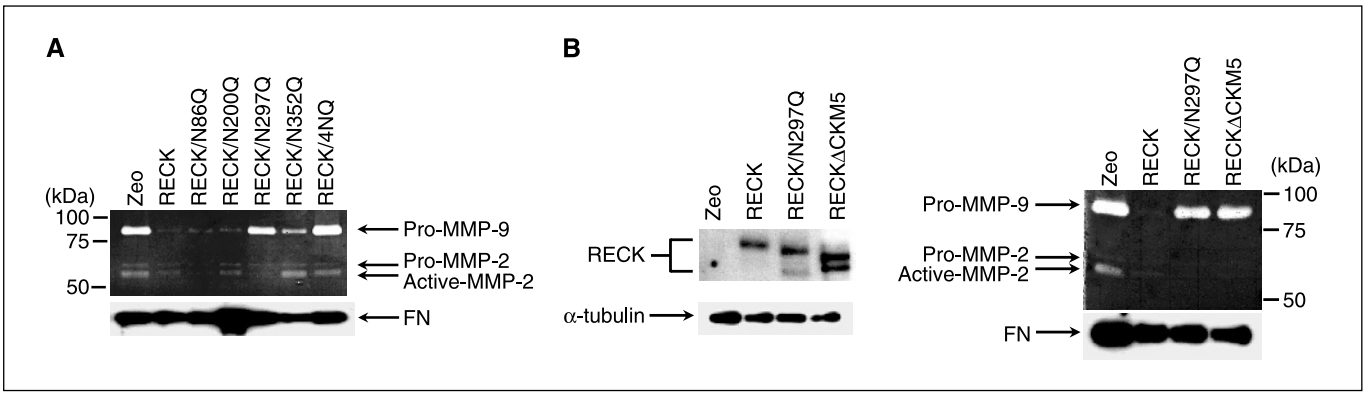

Figure 4. Glycosylation of RECK Asn^{297} and Asn^{352} residues is involved in the suppression of MMP-9 secretion and of MMP-2 activation, respectively. *A,* HT1080-Zeo, HT1080-RECK, HT1080-RECK/N86Q, HT1080-RECK/N200Q, HT1080-RECK/N297Q, HT1080-RECK/N352Q, and HT1080-RECK/4NQ cells were washed and cultured in serum-free medium for 24 hours. The prepared conditioned media from each cell line were analyzed by gelatin zymography. Fibronectin (*FN*) was monitored as the control for the secreted proteins in HT1080 cells, as measured by Western blotting using anti-fibronectin antibody. *B,* involvement of CKM5 region of RECK for suppression of MMP-9 secretion. Expression levels of RECK, RECK/N297Q, and RECK/ΔCKM5 were detected by Western blotting using indicated antibodies (*left*). HT1080-Zeo, HT1080-RECK, HT1080-RECK/N297Q, and HT1080-RECK/ΔCKM5 cells were washed and cultured in serum-free medium for 24 hours. The prepared conditioned media from each cell line were analyzed by gelatin zymography and Western blotting using anti-fibronectin antibody (*right*). Representative of three independent experiments.

medium (Fig. 4*A*). RECK/N86Q, RECK/N200Q, and RECK/N352Q also reduced the amount of MMP-9 in the culture media to the same level as that observed in association with wild-type RECK, indicating that the glycosylation of Asn^{86}, Asn^{200}, and Asn^{352} is not required for the suppression of MMP-9 secretion into culture media. On the other hand, RECK/N297Q and RECK/4NQ failed to suppress the secretion of MMP-9 into the culture media (Fig. 4*A*). Overexpression of RECK was also associated with a decrease in the amount of active MMP-2 in the conditioned medium (Fig. 4*A*). RECK/N86Q, RECK/N200Q, and RECK/N297Q also reduced the amount of active MMP-2 in the culture media, indicating that the glycosylation of Asn^{86}, Asn^{200}, and Asn^{297} is not required for the suppression of MMP-2 activation. On the other hand, RECK/N352Q and RECK/4NQ failed to suppress the activation of MMP-2 (Fig. 4*A*). The secretion levels of fibronectin, used as a control for secreted proteins in HT1080 cells, were not affected by the expression of wild-type and mutant RECK (Fig. 4*A*), suggesting that neither the expression of wild-type RECK proteins, nor that of these mutant RECK proteins, altered the general trafficking system in these cells. Thus, these findings indicate that the glycosylation of RECK Asn^{297} and Asn^{352} residues is involved in the suppression of MMP-9 secretion and of MMP-2 activation, respectively.

We showed that glycosylation of RECK at Asn^{297} is important for suppression of MMP-9 secretion (Fig. 4*A*), and that Asn^{297} is surely glycosylated by using concanavalin A-binding assay (Supplementary Fig. S1). Based on the primary amino acid sequence, it is thought that human RECK contains five cysteine knot motifs (Fig. 2*A*). Asn^{297} is the only glycosylation site located within a cysteine knot motif (amino acids, aa 292-338; designated as CKM5). To examine the role of CKM5 for suppression of MMP-9 secretion, we constructed the CKM5-deleted RECK (RECKΔCKM5) expression vector and established a RECKΔCKM5-expressing cell line (HT1080-RECKΔCKM5; Fig. 4*B*). The secretion of MMP-9 into the conditioned medium was not suppressed by RECKΔCKM5 expression, but MMP-2 activation was still inhibited (Fig. 4*B*). Thus, it is likely that the "N-glycosylated Asn^{297}-containing CKM5 region" is important for the suppression of MMP-9 secretion.

Circumvention of RECK-suppressed tumor cell invasion by inhibiting glycosylation of RECK. Because Takahashi et al. reported that the overexpression of RECK in HT1080 cells suppressed tumor cell invasion (14), we examined the effect of glycosylation on RECK-suppressed tumor cell invasion *in vitro*. As expected, the overexpression of RECK showed a suppression of invasion of ~40% (Fig. 5). HT1080-RECK/N200Q cells showed a suppression of the invasion; however, HT1080-RECK/N86Q, HT1080-RECK/N297Q, HT1080-RECK/N352Q, and HT1080-RECK/4NQ cells showed no such suppression of tumor cell invasion, as compared with HT1080-RECK cells (Fig. 5). It is thus possible that the circumvention of RECK-suppressed tumor cell invasion by a defect in glycosylation at Asn^{297} and at Asn^{352} was

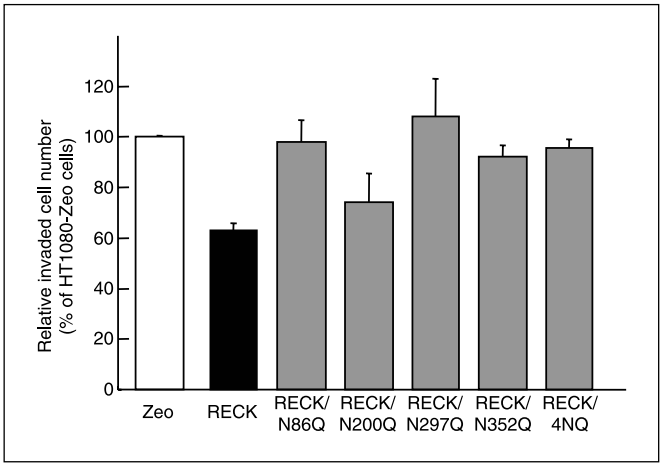

Figure 5. Circumvention of RECK-suppressed tumor cell invasion by inhibiting glycosylation of RECK. HT1080-Zeo, HT1080-RECK, HT1080-RECK/N86Q, HT1080-RECK/N200Q, HT1080-RECK/N297Q, HT1080-RECK/N352Q, or HT1080-RECK/4NQ cells were added to the upper chamber and incubated for 24 hours. Then the cells on the upper surface of the filter were completely removed by the use of cotton slabs, and the filters were fixed and stained with crystal violet. The dye was extracted, and the absorbance was measured at 540 nm. *Columns,* means of triplicate determinations; *bars,* ±SD. Representative of three independent experiments.

closely related to a failure to suppress the MMP-9 secretion into conditioned media and the MMP-2 activation, respectively. However, glycosylation at Asn^{86} residue regulated RECK function independent of suppressing MMP-9 secretion and inhibiting MMP-2 activation.

Discussion

The proteases (e.g., MMPs) and glycosidases (e.g., heparanase) play key roles in the normal physiology of connective tissue during development, morphogenesis, and wound healing, but their unregulated activity has been implicated in numerous disease processes including arthritis, tumor cell metastasis, and atherosclerosis (1–4, 9). Two important mechanisms regulating the activity of MMPs are (a) the binding of MMPs to a family of homologous proteins referred as the TIMPs and (b) the inhibition of both the enzymatic activity of MMPs and of their secretion into the extracellular space by RECK. RECK thus functions as a negative regulator of MMP activity by inhibiting secretion and activation (14, 15). Although the down-regulation of RECK has been reported in some human solid tumors, such as colorectal cancer (34–36), the posttranslational modification of RECK has not yet been investigated.

We first examined the expression of endogenous RECK in cultured human cell lines and observed the expression of RECK in nontransformed cell-derived lines including WI-38 and HUVECs, detected as two or three bands due to the difference in glycosylation status (Fig. 1*A*). Interestingly, RECK was also expressed in leukemia cell lines, such as U937 and Jurkat cells (Fig. 1*A*). It is possible that RECK has another function(s) in leukemia cells. Further studies will still be necessary to clarify the role(s) played by RECK in leukemia cells.

We showed that glycosylated RECK at the Asn^{297} residue is the important site for the suppression of MMP-9 secretion, whereas other glycosylation sites (i.e., Asn^{86}, Asn^{200}, and Asn^{352}) were not found to affect the MMP-9 secretion (Fig. 4*A*). Based on the primary amino acid sequence, it is thought that human RECK contains five cysteine knot motifs (aa 37-84, aa 104-141, aa 151-197, aa 216-263, and aa 292-338, respectively: cf. Fig. 2*A*). Asn^{297} is the only glycosylation site located within a cysteine knot motif, CKM5. The cysteine knot motif is found in extracellular fragments of signaling proteins including the Met receptor (37), platelet glycoprotein Ibβ (GPIbβ; ref. 38), the choriogonadotropin β-subunit (39), and transforming growth factor-β (40). In the case of GPIbβ, the cysteine knot region in the NH_2 terminus is critical for the conformation of GPIbβ, which interacts with another protein (38). Therefore, we had speculated that CKM5 region might be critical for the suppression of MMP-9 secretion by RECK. To this end, we established a RECKΔCKM5-expressing cell line (HT1080-RECKΔCKM5). The secretion of MMP-9 into the conditioned medium was not suppressed by RECKΔCKM5 expression (Fig. 4*B*). Thus, it is likely that the "N-glycosylated Asn^{297}-containing CKM5 region" is important for the suppression of MMP-9 secretion and also for the inhibition of tumor cell invasion (data not shown). Further studies are needed to determine why the deletion of the CKM5 region results in the inactivation of RECK, which seems to induce the suppression of MMP-9 secretion.

It has been reported that RECK can inhibit not only the secretion of MMP-9 but is also capable of directly inhibiting the activity of MMP-2 and membrane-type 1 MMP (MT1-MMP; refs. 14, 15). In this study, we showed that glycosylation at Asn^{352} residue is important for the suppression of MMP-2 activation; however, RECK also has other possible functional regions (e.g., three protease inhibitor-like domains; Kazal-like motifs). The first of these domains (aa 635-654) completely matches the Kazal motif, whereas the second (aa 716-735) and the third (aa 754-772) define incomplete Kazal motifs (14). It is possible that these three Kazal-like motifs may be also responsible for the inhibition of MMP-2 and MT1-MMP proteolytic activities. At present, it would be premature to discuss the role played by glycosylation in RECK's antagonism of the inhibitory activities of MMP-2 and MT1-MMP. Furthermore, RECK/N86Q could suppress MMP-9 secretion and MMP-2 activation; however, RECK/N86Q did not suppress the invasion (Fig. 5). Thus, it is suggested that RECK functions as an inhibitor of tumor cell invasion by multiple mechanisms such as suppressing MMP-9 secretion, inhibiting MMP-2 activation, and unknown mechanism. The identification of the role for Asn^{86} glycosylation will enhance our general understanding of the important roles played by RECK.

Because RECK expression is down-regulated in many tumor types (34–36), several groups have attempted to obtain agents with the ability to up-regulate RECK expression in human tumor cells. Using a RECK promoter-luciferase system, Liu et al. identified some drugs, such as trichostatin A (41), a histone deacetylase inhibitor, and a nonsteroidal anti-inflammatory, both of which up-regulated RECK in tumor cell lines (42, 43). Treatment of tumor cells with each of these drugs resulted in an induction of RECK expression and in the inactivation of MMPs, thereby inhibiting tumor cell invasion (42, 43). Inhibition of RECK expression by small interference RNA abolished the inhibitory effect of trichostatin A on MMP activation (42). Thus, it is reasonable to expect that RECK, as a target, is responsible for trichostatin A and nonsteroidal anti-inflammatory inhibition of tumor cell invasion. Furthermore, it has been reported that EBV latent membrane protein 1 (LMP1) induced RECK down-regulation via the repression of transcription (44). Treatment with PD98059 antagonized the LMP1-induced down-regulation of RECK (44), suggesting that this type of repression was required for the extracellular signal-regulated kinase (ERK) signal pathway. The inhibitors of the ERK-signaling pathway might be effective antimetastasis agents in patients with EBV-positive cancers such as nasopharyngeal carcinoma.

These results, when taken together, suggest that hyperglycosylation/hypoglycosylation is an important key to the function of RECK; drugs able to induce RECK expression may provide effective therapy and/or act as novel chemosensitizers for the treatment of human cancer cell lines that do not express RECK.

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